INSECT SODIUM CHANNELS FROM INSECTICIDE-SUSCEPTIBLE AND INSECTICIDE-RESISTANT HOUSE FLIES

The subject matter of this application was made with support from the United States Government under USDA Grant No. 94-37302-0408.

This application is a continuation-in-part of U.S. Serial No. 08/608,618, filed March 1, 1996, the contents of which are hereby incorporated by reference.

FIELD OF THE INVENTION

The present invention relates generally to insect sodium channel proteins, and more particularly to insecticide-susceptible and insecticide-resistant voltage-sensitive sodium channels of the house fly *Musca domestica*.

BACKGROUND OF THE INVENTION

Throughout this application various publications are referenced, many in parenthesis. Full citations for these publications are provided at the end of the Detailed Description. The disclosures of these publications in their entireties are hereby incorporated by reference in this application.

Cell membranes must allow passage of various polar molecules, including ions, sugars, amino acids, and nucleotides. Special membrane proteins are responsible for transferring such molecules across cell membranes. These proteins, referred to as membrane transport proteins, occur in many forms and in all types of biological membranes. Each protein is specific in that it transports a particular class of molecules (such as ions, sugars, or amino acids) and often only certain molecular species of the class. All membrane transport proteins that have been studied in detail have been found to be multipass transmembrane proteins. By forming a continuous

protein pathway across the membrane, these proteins enable the specific molecules to cross the membrane without coming into direct contact with the hydrophobic interior of the lipid bilayer of the plasma membrane.

There are two major classes of membrane transport proteins: carrier proteins and channel proteins. Carrier proteins bind the specific molecule to be transported and undergo a series of conformational changes in order to transfer the bound molecule across the membrane. Channel proteins, on the other hand, need not bind the molecule. Instead, they form hydrophilic pores that extend across the lipid bilayer; when these pores are open, they allow specific molecules (usually inorganic ions of appropriate size and charge) to pass through them and thereby cross the membrane. Transport through channel proteins occurs at a much faster rate than transport mediated by carrier proteins.

Channel proteins which are concerned specifically with inorganic ion transport are referred to as ion channels, and include ion channels for sodium, potassium, calcium, and chloride ions. Ion channels which open in response to a change in the voltage across the membrane are referred to as voltage-sensitive ion channels.

The sodium channel is one of the most thoroughly characterized of the voltage-sensitive channels (see Fig. 1 for a model of a voltage-sensitive sodium channel). In vertebrates, sodium channels in the brain, muscle, and other tissues are large membrane glycoprotein complexes composed of an alpha subunit (230-270 kDa) and 1-2 tightly associated smaller (33-38 kDa) beta subunits (reviewed by Catterall 1992). The large alpha subunit forms the ion permeable pore while the smaller subunits play key roles in the regulation of channel function (Isom et al. 1992; reviewed by Isom et al. 1994). The alpha

subunit is common to purified channel preparations from *Electrophorus electricus* (electric eel) electric organ (Noda et al. 1984), rat brain (Noda et al. 1986), rat skeletal muscle (Barchi 1988) and chick heart muscle (Catterall 1986). Other studies have revealed the existence of multiple closely related isoforms of the sodium channel found in different animal species, in different tissues within the same species, and even in the same tissue (Catterall et al. 1981; Frelin et al. 1984; Rogart 1986; Moczydlowski et al. 1986).

The structure of invertebrate sodium channels is not as well defined. Gene cloning studies have established the existence of alpha subunits of structure similar to those described for vertebrates (Loughney et al. 1989; Ramaswami and Tanouye 1989; Okamoto et al. 1987). Analysis of the *para* behavioral mutant (paralytic; Suzuki et al. 1971) of *Drosophila melanogaster* revealed that the *para* gene encodes a *Drosophila* sodium channel alpha subunit (Loughney et al. 1989). The entire *para* cDNA sequence was determined (Loughney et al. 1989; Thackeray and Ganetzky 1994).

The *kdr* mutant of the house fly *Musca domestica* has also been studied. The *kdr* insecticide resistance trait of the house fly confers reduced neuronal sensitivity to the rapid paralytic and lethal actions of DDT and pyrethroid insecticides (Soderlund and Bloomquist 1990). Because these insecticides are known to modify neuronal excitability by altering the inactivation kinetics of voltage-sensitive sodium channels (Soderlund and Bloomquist 1989; Bloomquist 1993), efforts to identify the molecular basis of *kdr* resistance have focused on the pharmacology and structure of this target.

Recently, tight genetic linkage between the *kdr* trait and a restriction fragment length polymorphism located within a segment of the house fly homolog of the

para gene of *Drosophila melanogaster* was demonstrated (Knipple et al. 1994). Similar linkage studies have also documented tight linkage of the *super-kdr* resistance trait of the house fly (Williamson et al. 1993) to molecular markers lying within the *para*-homologous voltage-sensitive sodium channel gene.

Elucidation of the structure of the house fly sodium channel gene will enable the screening of potential insecticidal agents which act upon the sodium channel.

A need continues to exist, therefore, for the determination of the primary structure of the house fly sodium channel, i.e. the nucleotide and amino acid sequences of the channel.

SUMMARY OF INVENTION

To this end, the subject invention provides the 6318 nucleotide coding sequence (SEQ ID NO:1) of the voltage-sensitive sodium channel gene from insecticide-susceptible (NAIDM strain) house flies (*Musca domestica*), determined by automated direct DNA sequencing of PCR fragments obtained by amplification on first strand cDNA from adult heads. The deduced 2105-residue amino acid sequence (SEQ ID NO:3) exhibits overall structure and organization typical of sodium channel alpha subunit genes and is 90.0% identical to that of the *D. melanogaster para* gene product. There is no evidence for the existence of multiple splice variants among voltage-sensitive sodium channel cDNAs obtained from adult house fly head preparations. Comparison of the coding sequence of the voltage-sensitive sodium channel gene of the *kdr* insecticide-resistant house fly strain (538ge strain) to that of the NAIDM strain reveals 12 amino acid differences in the 538ge strain. The amino acid sequence (SEQ ID NO:4) of the *Kdr* strain is only 2104 residues in length, as a result of five (5) amino acid substitutions, four (4)

amino acid deletions, and three (3) amino acid insertions as compared to the 2105-residue amino acid sequence (SEQ ID NO:3) of the NAIDM strain. The nucleotide sequence (SEQ ID NO:2) of the *Kdr* strain is therefore 6315 nucleotides in length, which is three nucleotides shorter than the nucleotide sequence (SEQ ID NO:1) of the NAIDM strain.

More particularly, the subject invention provides an isolated nucleic acid molecule encoding a voltage-sensitive sodium channel of *Musca domestica*, wherein the voltage-sensitive sodium channel is capable of conferring sensitivity or resistance to an insecticide in *Musca domestica*. In one embodiment, the nucleic acid molecule confers insecticide susceptibility to the house fly, and in another embodiment the nucleic acid molecule confers insecticide resistance to the house fly. The nucleic acid molecule conferring insecticide resistance is preferably a mutated form of the nucleic acid molecule encoding the insecticide susceptible channel. The invention also provides an antisense nucleic acid molecule complementary to mRNA encoding the voltage-sensitive sodium channel of *Musca domestica*.

The isolated nucleic acid molecules of the invention can be inserted into suitable expression vectors and/or host cells. Expression of the nucleic acid molecules encoding the sodium channels results in production of functional sodium channels in a host cell. Expression of the antisense nucleic acid molecules or fragments thereof in a host cell results in decreased expression of the functional sodium channels.

The invention further provides a ribozyme having a recognition sequence complementary to a portion of mRNA encoding a voltage-sensitive sodium channel of *Musca domestica*. The ribozyme can be introduced into a

cell to also achieve decreased expression of sodium channels in the cell.

The invention further provides a method of screening a chemical agent for the ability of the chemical agent to modify sodium channel function, and a method of obtaining DNA encoding a voltage-sensitive sodium channel of *Musca domestica*.

Further provided is an isolated nucleic acid molecule encoding a voltage-sensitive sodium channel of an insect, wherein the nucleic acid molecule encodes a first amino acid sequence having at least 95% amino acid identity to a second amino acid sequence. The second amino acid sequence is, in two preferred embodiments, SEQ ID NO:3 or SEQ ID NO:4.

The invention also provides an isolated voltage-sensitive sodium channel of *Musca domestica*, and antibodies or antibody fragments specific for the sodium channel. The antibodies or antibody fragments can be used to detect the presence of the sodium channel in samples.

Further provided is an isolated voltage-sensitive sodium channel of *Musca domestica*, wherein the voltage-sensitive sodium channel is comprised of a protein having a first amino acid sequence with at least 95% amino acid identity to a second amino acid sequence. In two preferred embodiments, the second amino acid sequence is SEQ ID NO:3 or SEQ ID NO:4.

Also provided by the subject invention is a plasmid designated pPJI1 and deposited with the ATCC under Accession No. 97831, as well as a KpnI/AatII restriction fragment of about 3620 bp of the plasmid designated pPJI1. Further provided is a plasmid designated pPJI2 and deposited with the ATCC under Accession No. 97852, as well as an AatII/SphII restriction fragment of about 2700 bp of the plasmid designated pPJI2. When the above two restriction fragments are ligated together at their AatII

sites, the resulting nucleic acid molecule encodes a voltage-sensitive sodium channel which confers susceptibility to an insecticide in *Musca domestica*. This resulting nucleic acid molecule is also provided by the subject invention.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features and advantages of this invention will be evident from the following detailed description of preferred embodiments when read in conjunction with the accompanying drawings in which:

Fig. 1 is a model of a voltage sensitive sodium channel from mammalian brain in the plasma membrane. The alpha and beta$_1$ subunits interact noncovalently; the alpha and beta$_2$ subunits are linked by disulfide bonds. The branched structures at the outer surface of the channel represent oligosaccharides;

Fig. 2 is a diagram of the structural organization of the voltage-sensitive sodium channel coding sequence of *Musca domestica* (*Vssc1*) showing repeated homology domains I-IV and putative transmembrane helices (rectangles). Shown below the structural organization are the relative length and location of the previously-described 309-nucleotide exon of *Vssc1* (Knipple *et al.* 1994) (exon) and seven overlapping PCR-amplified cDNA fragments (A-G) employed as templates for DNA sequencing;

Fig. 3 shows the alignment of the predicted amino acid sequences of *Vssc1*^{NAIDM} (NAIDM) (SEQ ID NO:3) and *Vssc1*^{538ge} (538ge) (SEQ ID NO:4) with that of the *a*$^+$*b*$^-$*c*$^-$*d*$^+$*e*$^-$*f*$^-$*h*$^-$*i*$^+$ splice variant of the *D. melanogaster para* sequence (para) obtained using the DNASTAR computer program (Clustal method). Residues that are identical to the NAIDM sequence in both 538ge and para are indicated as dashes (-) in the latter two sequences; gaps introduced to obtain optimal alignment are

indicated as periods (.). The locations of 24 putative helical transmembrane domains (e.g., IS1, IS2, etc.) and four putative pore-forming domains (e.g., IP, IIP) are marked by solid bars above the NAIDM sequence. Also marked above the NAIDM sequence are possible sites for *N*-linked glycosylation (#), cAMP-dependent protein kinase phosphorylation (*), and protein kinase C phosphorylation (o); and

Fig. 4 is a diagram of the *Vssc1* gene product showing the locations of 12 amino acid differences identified in the *Vssc1*^{538ge} sequence, including 5 amino acid substitutions, 4 amino acid deletions, and 3 amino acid insertions in the Vssc1^{538ge} sequence (R) as compared to the Vssc1^{NAIDM} sequence (S).

DETAILED DESCRIPTION

The plasmids designated pPJI1 and pPJI2 have each been deposited pursuant to, and in satisfaction of, the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, 20852 under ATCC Accession No. 97831 (pPJI1) and ATCC Accession No. 97831 (pPJI2). Both deposits were made on December 20, 1996.

As used herein, the term "isolated" when used in conjunction with a nucleic acid molecule refers to: 1) a nucleic acid molecule which has been separated from an organism in a substantially purified form (i.e. substantially free of other substances originating from that organism), or 2) a nucleic acid molecule having the same nucleotide sequence but not necessarily separated from the organism (i.e. synthesized nucleic acid molecules). The term "isolated" when used in conjunction with a channel refers to a channel encoded by such an

"isolated" nucleic acid molecule, generally expressed in a membrane, such as a plasma membrane within a cell or a synthetic lipid bilayer membrane. The expressed "isolated" channel has the pharmacological properties of a functional sodium channel.

As further used herein, the terms "corresponding to" or "having" or "as shown in" when used in conjunction with a SEQ ID NO for a nucleotide sequence refer to a nucleotide sequence which is substantially the same nucleotide sequence, or derivatives or equivalents thereof (such as deletion and hybrid variants thereof, splice variants thereof, etc.). Nucleotide additions, deletions, and/or substitutions, such as those which do not affect the translation of the DNA molecule, are within the scope of a nucleotide sequence corresponding to or having or as shown in a particular nucleotide sequence (i.e. the amino acid sequence encoded thereby remains the same). Such additions, deletions, and/or substitutions can be, for example, point mutations made according to methods known to those skilled in the art. It is also possible to substitute a nucleotide which alters the amino acid sequence encoded thereby, where the amino acid substituted is a conservative substitution or where amino acid homology is conserved. It is also possible to have minor nucleotide additions, deletions, and/or substitutions which do not alter the function of the resulting VSSC. Similarly, the term "corresponding to" or "having" or "as shown in" when used in conjunction with a SEQ ID NO for an amino acid sequence refers to an amino acid sequence which is substantially the same amino acid sequence or derivatives or equivalents thereof. Amino acid additions, deletions, and/or substitutions which do not negate the ability of the resulting protein to form a functional sodium channel are within the scope of an amino acid sequence corresponding to or having or as shown in a

particular amino acid sequence. Such additions, deletions, and/or substitutions can be, for example, the result of point mutations in the DNA encoding the amino acid sequence, such point mutations made according to methods known to those skilled in the art. Substitutions may be conservative substitutions of amino acids. As used herein, two amino acid residues are conservative substitutions of one another where the two residues are of the same type. In this regard, for purposes of the present invention, proline, alanine, glycine, serine, and threonine, all of which are neutral, weakly hydrophobic residues, are of the same type. Glutamine, glutamic acid, asparagine, and aspartic acid, all of which are acidic, hydrophilic residues, are of the same type. Another type of residue is the basic, hydrophilic amino acid residues, which include histidine, lysine, and arginine. Leucine, isoleucine, valine, and methionine all of which are hydrophobic, aliphatic amino acid residues, form yet another type of residue. Yet another type of residue consists of phenylalanine, tyrosine, and tryptophan, all of which are hydrophobic, aromatic residues. Further descriptions of the concept of conservative substitutions are given by French and Robson 1983, Taylor 1986, and Bordo and Argos 1991.

As further used herein, the term "corresponding to" or "having" or "as shown in" or "consisting of" when used in conjunction with a SEQ ID NO for a nucleotide or amino acid sequence is intended to cover linear or cyclic versions of the recited sequence (cyclic referring to entirely cyclic versions or versions in which only a portion of the molecule is cyclic, including, for example, a single amino acid cyclic upon itself), and is intended to cover derivative or modified nucleotides or amino acids within the recited sequence. For example, those skilled in the art will readily understand that an adenine

nucleotide could be replaced with a methyladenine, or a cytosine nucleotide could be replaced with a methylcytosine, if a methyl side chain is desirable. Nucleotide sequences having a given SEQ ID NO are intended to encompass nucleotide sequences containing these and like derivative or modified nucleotides, as well as cyclic variations. As a further example, those skilled in the art will readily understand that an asparagine residue could be replaced with an ethylasparagine if an ethyl side chain is desired, a lysine residue could be replaced with a hydroxylysine if an OH side chain is desired, or a valine residue could be replaced with a methylvaline if a methyl side chain is desired. Amino acid sequences having a given SEQ ID NO are intended to encompass amino acid sequences containing these and like derivative or modified amino acids, as well as cyclic variations. Cyclic, as used herein, also refers to cyclic versions of the derivative or modified nucleotides and amino acids.

The function of the encoded sodium channel can be assayed according to methods known in the art, such as by voltage clamp analysis of the channel following the functional expression of the channel in oocytes of the frog *Xenopus laevis* (see Taglialatela et al. 1992 and Stuhmer 1992 for a general discussion of the voltage clamp analysis of receptors and ion channels expressed in *Xenopus* oocytes). As used herein, "functional expression" refers to the synthesis and any necessary post-translational processing of a sodium channel molecule in a host cell so that the channel is inserted properly in the cell membrane and is capable of conducting sodium ions in response to an experimentally-imposed change in the cell membrane potential or upon exposure to appropriate pharmacological agents.

As further used herein, "sensitivity" and "resistance" refer to the relative responses of

genetically-defined insect populations to the paralytic or lethal actions of a test insecticide. For example, a dose of DDT [1,1-bis-(4-chlorophenyl)-2,2,2-trichloroethane] of approximately 0.02 μg per adult fly will kill approximately 50% of the treated individuals of a susceptible (Cooper-S) house fly strain, whereas doses of approximately 0.5 μg per adult fly are required to kill approximately 50% of the treated individuals of a resistant (538ge) house fly strain (Sawicki 1978). The absolute doses that define susceptibility and resistance vary with the insect species and genetically defined populations examined, the test insecticide employed, and the method of exposure. In general, an insect strain or population is considered "resistant" if it exhibits tolerance to a test insecticide (assessed as the dose required to poison 50% of a treated population or group) that is at least 10 times greater than the tolerance of an appropriate reference, or "susceptible" population. Test insecticides include not only DDT but also analogs of DDT (e.g., methoxychlor, perthane) and pyrethroid insecticides (e.g., deltamethrin, fenvalerate, resmethrin, permethrin).

As also used herein, insects include *Musca domestica* (the house fly), the fruit or vinegar fly (*Drosophila melanogaster*), and various other insect species of agricultural, medical or veterinary importance, such as *Heliothis virescens* (the tobacco budworm), *Leptinotarsa decemlineata* (the Colorado potato beetle), *Blattella germanica* (the German cockroach), and *Aedes aegypti* (the yellow fever mosquito).

The subject invention provides an isolated nucleic acid molecule encoding a voltage-sensitive sodium channel (VSSC) of *Musca domestica*, wherein the VSSC is capable of conferring sensitivity or resistance to an insecticide in *Musca domestica*. The nucleic acid molecule can be deoxyribonucleic acid (DNA) or ribonucleic acid

(RNA, including messenger RNA or mRNA), genomic or recombinant, biologically isolated or synthetic.

The DNA molecule can be a cDNA molecule, which is a DNA copy of a messenger RNA (mRNA) encoding the VSSC.

In one embodiment, the VSSC confers insecticide susceptibility to *Musca domestica*. An example of such an insecticide susceptible VSSC is the channel encoded by the nucleotide sequence as shown in SEQ ID NO:1. SEQ ID NO:1 is the DNA sequence of one allele of the VSSC of *Musca domestica*. The amino acid sequence encoded by this allele is shown in SEQ ID NO:3.

In another embodiment, the VSSC confers insecticide resistance to *Musca domestica*. An example of such an insecticide resistant VSSC is the channel encoded by the nucleotide sequence as shown in SEQ ID NO:2. SEQ ID NO:2 is the DNA sequence of another allele of the VSSC of *Musca domestica* characteristic of the kdr insecticide resistant strain. The amino acid sequence encoded by this mutant allele is shown in SEQ ID NO:4.

The insecticide resistant allele preferably has the nucleotide sequence of a second nucleic acid molecule with one or more mutations therein, wherein the second nucleic acid molecule encodes an insecticide sensitive VSSC and wherein one or more mutations in the second nucleic acid molecule render the resulting VSSC resistant to an insecticide (hence the term "mutant" allele). In one embodiment, the mutant allele (having amino acid SEQ ID NO:4) has the amino acid sequence encoded by the susceptibility allele (amino acid SEQ ID NO:3) with amino acid differences as follows: a substitution of phenylalanine for leucine at amino acid residue 1014 of SEQ ID NO:3; a substitution of isoleucine for methionine at amino acid residue 1140 of SEQ ID NO:3; a substitution of aspartic acid for glycine at amino acid residue 2023 of SEQ ID NO:3; a deletion of amino acid residues 2031-2034

of SEQ ID NO:3 (glycine-alanine-threonine-alanine); a substitution of threonine for serine at amino acid residue 2042 of SEQ ID NO:3; a substitution of alanine for valine at amino acid residue 2054 of SEQ ID NO:3; and an insertion of three amino acid residues (asparagine-glycine-glycine) after amino acid residue 2055 of SEQ ID NO:3 (between amino acid residues 2055 and 2056 of SEQ ID NO:3). One or more of these amino acid differences can be included in an insecticide resistant VSSC. Other suitable sites for mutations can be identified by conventional, molecular genetic approaches, such as the identification of amino acid sequence substitutions/insertions/deletions in the VSSC sequences of other insecticide-resistant house fly strains.

The invention also provides an antisense nucleic acid molecule that is complementary to the mRNA encoding the VSSC, or a fragment thereof. Antisense nucleic acid molecules can be RNA or single-stranded DNA. Antisense molecules can be complementary to the entire DNA molecule encoding the VSSC, i.e. of the same nucleotide length as the entire molecule. It may be desirable, however, to work with a shorter molecule. In this instance, fragments of the entire antisense molecule can be used. Suitable fragments are capable of hybridizing to the mRNA encoding the entire molecule, and preferably consist of at least twenty nucleotides. These antisense molecules and fragments thereof can be used to reduce steady state levels of a VSSC gene product of *Musca domestica*, by introducing into cells an RNA or single-stranded DNA molecule that is complementary to the mRNA of the VSSC (i.e. by introducing an antisense molecule). The antisense molecule can base-pair with the mRNA of the VSSC, preventing translation of the mRNA into protein. Thus, an antisense molecule to the VSSC of *Musca domestica*

can prevent translation of mRNA encoding the VSSC into a functional sodium channel protein.

More particularly, an antisense molecule complementary to mRNA encoding a VSSC of *Musca domestica*, or a fragment thereof, can be used to decrease expression of a functional VSSC of *Musca domestica*. A cell with a first level of expression of a functional VSSC of *Musca domestica* is first selected, and then the antisense molecule (or fragment thereof) is introduced into the cell. The antisense molecule (or fragment thereof) blocks expression of functional VSSCs of *Musca domestica*, resulting in a second level of expression of a functional VSSC of *Musca domestica* in the cell. The second level is less than the initial first level.

Antisense molecules can be introduced into cells by any suitable means. Suitable cells include *Xenopus* oocytes which are useful host cells for studying the expression of the encoded sodium channel, and various insect cells, including but not limited to the insect cell lines *Drosophila Schneider* (Johansen et al. 1989), *Drosophila* K_c (Sang 1981), Sf9 (Smith et al. 1983), and High Five® (see U.S. Patent No. 5,300,435). In one embodiment, the antisense RNA molecule is injected directly into the cellular cytoplasm, where the RNA interferes with translation. A vector may also be used for introduction of the antisense molecule into a cell. Such vectors include various plasmid and viral vectors. For a general discussion of antisense molecules and their use, see Han et al. 1991 and Rossi 1995.

The invention further provides a special category of antisense RNA molecules, known as ribozymes, having recognition sequences complementary to specific regions of the mRNA encoding the VSSC of *Musca domestica*. Ribozymes not only complex with target sequences via complementary antisense sequences but also catalyze the

hydrolysis, or cleavage, of the template mRNA molecule. Examples, which are not intended to be limiting, of suitable regions of the mRNA template to be targeted by ribozymes are any of the regions encoding the 24 putative transmembrane domains of the VSSC of *Musca domestica*.

Expression of a ribozyme in a cell can inhibit gene expression (such as the expression of a VSSC of *Musca domestica*). More particularly, a ribozyme having a recognition sequence complementary to a region of a mRNA encoding a VSSC of *Musca domestica* can be used to decrease expression of a functional VSSC of *Musca domestica*. A cell with a first level of expression of a functional VSSC of *Musca domestica* is first selected, and then the ribozyme is introduced into the cell. The ribozyme in the cell decreases expression of a functional VSSC of *Musca domestica* in the cell, because mRNA encoding the VSSC is cleaved and cannot be translated.

Ribozymes can be introduced into cells by any suitable means. Suitable cells include *Xenopus* oocytes which are useful host cells for studying the expression of the encoded sodium channel, and various insect cells, including but not limited to the insect cell lines *Drosophila Schneider*, *Drosophila* K_c, Sf9, and High Five®. In one embodiment, the ribozyme is injected directly into the cellular cytoplasm, where the ribozyme cleaves the mRNA and thereby interferes with translation. A vector may be used for introduction of the ribozyme into a cell. Such vectors include various plasmid and viral vectors (note that the DNA encoding the ribozyme does not need to be "incorporated" into the genome of the host cell; it could be expressed in a host cell infected by a viral vector, with the vector expressing the ribozyme, for instance). For a general discussion of ribozymes and their use, see Sarver et al. 1990, Chrisey et al. 1991, Rossi et al. 1992, and Christoffersen et al. 1995.

The nucleic acid molecules of the subject invention can be expressed in suitable host cells using conventional techniques. Any suitable host and/or vector system can be used to express the VSSCs. These include, but are not limited to, eukaryotic hosts such as mammalian cells (i.e., Hela cells, Cv-1 cells, COS cells), *Xenopus* oocytes, and insect cells (i.e. insect cell lines such as *Drosophila Schneider, Drosophila* K_c, Sf9, and High Five®).

Techniques for introducing the nucleic acid molecules into the host cells may involve the use of expression vectors which comprise the nucleic acid molecules. These expression vectors (such as plasmids and viruses; viruses including bacteriophage) can then be used to introduce the nucleic acid molecules into suitable host cells. For example, sodium channel expression is often studied in *Xenopus* oocytes. DNA encoding the VSSC can be injected into the oocyte nucleus or transformed into the oocyte using a suitable vector, or mRNA encoding the VSSC can be injected directly into the oocyte, in order to obtain expression of a functional VSSC in the oocyte. It may be beneficial when expressing the sodium channels of the subject invention in *Xenopus* oocytes to coexpress a nucleic acid molecule encoding a tipE protein (Feng et al. 1995). Tip E has been found to be necessary to obtain expression of some sodium channels in *Xenopus* oocytes (Feng et al. 1995).

Various methods are known in the art for introducing nucleic acid molecules into host cells. One method is microinjection, in which DNA is injected directly into the nucleus of cells through fine glass needles (or RNA is injected directly into the cytoplasm of cells). Alternatively, DNA can be incubated with an inert carbohydrate polymer (dextran) to which a positively charged chemical group (DEAE, for diethylaminoethyl) has been coupled. The DNA sticks to the DEAE-dextran via its

negatively charged phosphate groups. These large DNA-containing particles stick in turn to the surfaces of cells, which are thought to take them in by a process known as endocytosis. Some of the DNA evades destruction in the cytoplasm of the cell and escapes to the nucleus, where it can be transcribed into RNA like any other gene in the cell. In another method, cells efficiently take in DNA in the form of a precipitate with calcium phosphate. In electroporation, cells are placed in a solution containing DNA and subjected to a brief electrical pulse that causes holes to open transiently in their membranes. DNA enters through the holes directly into the cytoplasm, bypassing the endocytotic vesicles through which they pass in the DEAE-dextran and calcium phosphate procedures (passage through these vesicles may sometimes destroy or damage DNA). DNA can also be incorporated into artificial lipid vesicles, liposomes, which fuse with the cell membrane, delivering their contents directly into the cytoplasm. In an even more direct approach, used primarily with plant cells and tissues, DNA is absorbed to the surface of tungsten microprojectiles and fired into cells with a device resembling a shotgun.

Several of these methods, microinjection, electroporation, and liposome fusion, have been adapted to introduce proteins into cells. For review, see Mannino and Gould-Fogerite 1988, Shigekawa and Dower 1988, Capecchi 1980, and Klein et al. 1987.

Further methods for introducing nucleic acid molecules into cells involve the use of viral vectors. Since viral growth depends on the ability to get the viral genome into cells, viruses have devised clever and efficient methods for doing it. One such virus widely used for protein production is an insect virus, baculovirus. Baculovirus attracted the attention of researchers because during infection, it produces one of

its structural proteins (the coat protein) to spectacular levels. If a foreign gene were to be substituted for this viral gene, it too ought to be produced at high level. Baculovirus, like vaccinia, is very large, and therefore foreign genes must be placed in the viral genome by recombination. To express a foreign gene in baculovirus, the gene of interest is cloned in place of the viral coat protein gene in a plasmid carrying a small portion of the viral genome. The recombinant plasmid is cotransfected into insect cells with wild-type baculovirus DNA. At a low frequency, the plasmid and viral DNAs recombine through homologous sequences, resulting in the insertion of the foreign gene into the viral genome. Virus plaques develop, and the plaques containing recombinant virus look different because they lack the coat protein. The plaques with recombinant virus are picked and expanded. This virus stock is then used to infect a fresh culture of insect cells, resulting in high expression of the foreign protein. For a review of baculovirus vectors, see Miller (1989). Various viral vectors have also been used to transform mammalian cells, such as bacteriophage, vaccinia virus, adenovirus, and retrovirus.

As indicated, some of these methods of transforming a cell require the use of an intermediate plasmid vector. U.S. Patent No. 4,237,224 to Cohen and Boyer describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture. The DNA sequences are cloned into the plasmid vector using standard cloning procedures known in the art, as described by Sambrook et al. (1989).

Host cells into which the nucleic acid encoding the VSSC has been introduced can be used to produce (i.e. to functionally express) the voltage-sensitive sodium channel.

Having identified the nucleic acid molecules encoding VSSCs and methods for expressing functional channels encoded thereby, the invention further provides a method of screening a chemical agent for the ability of the chemical agent to modify sodium channel function. The method comprises introducing a nucleic acid molecule encoding the VSSC into a host cell, and expressing the VSSC encoded by the molecule in the host cell. The expression results in the functional expression of a VSSC in the membrane of the host cell. The cell is then exposed to a chemical agent and evaluated to determine if the chemical agent modifies the function of the VSSC. From this evaluation, chemical agents effective in altering the function of the sodium channel can be found. Such agents may be, for example, tetrodotoxin, veratridine, and scorpion venom toxins. Additional agents can be found in Soderlund and Knipple 1994.

Cells transformed to include the VSSC according to the subject invention can be exposed to various potential insecticides and pesticides and evaluated for their susceptibility to the agents to develop and identify insect control agents that will not cause adverse effects to vertebrate species. Exemplary methods of screening are described in Eldefrawi et al. 1987 and Rauh et al. 1990. The evaluation of the function of the sodium channel can be by any means known in the art. In one embodiment, the evaluation comprises monitoring sodium transport through the VSSC. Sodium transport can be monitored by pre-incubating cells in a medium containing one or more chemical agents, adding a medium containing radiosodium ($^{22}Na^{+}$), incubating the cells further in this medium, and

isolating cells by filtration. Sodium transport is detected by the measurement of $^{22}Na^{+}$ within the cells by liquid scintillation counting or other radiometric techniques (Bloomquist and Soderlund 1988). Alternatively, [^{14}C]guanidinium ion can be employed as the radiotracer in the place of sodium using the same procedure (Jacques et al. 1978). In another embodiment, the function of the VSSC can be evaluated by pre-incubating cells to equilibrium with a sodium-selective fluorescent chelating agent (e.g., SBFI [sodium-binding benzofuran isophthalate]), washing the cells, exposing the cells to a test agent, and monitoring the increase in intracellular sodium by measuring the fluorescence of the SBFI-sodium complex (Deri and Adam-Vizi 1993).

The nucleic acid molecules of the subject invention can be used either as probes or for the design of primers to obtain DNA encoding other VSSCs by either cloning and colony/plaque hybridization or amplification using the polymerase chain reaction (PCR).

Specific probes derived from SEQ ID NOs 1 or 2 can be employed to identify colonies or plaques containing cloned DNA encoding a member of the VSSC family using known methods (see Sambrook et al. 1989). One skilled in the art will recognize that by employing such probes under high stringency conditions (for example, hybridization at 42°C with 5X SSPC and 50% formamide, washing at 50-65°C with 0.5X SSPC), sequences having regions which are greater than 90% identical to the probe can be obtained. Sequences with lower percent identity to the probe, which also encode VSSCs, can be obtained by lowering the stringency of hybridization and washing (for example, by reducing the hybridization and wash temperatures or reducing the amount of formamide employed).

More particularly, in one embodiment, the method comprises selection of a DNA molecule encoding a

VSSC of an insect, or a fragment thereof, the DNA molecule having a nucleotide sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2, and designing an oligonucleotide probe for a VSSC based on SEQ ID NO:1 or SEQ ID NO:2. A genomic or cDNA library of an insect is then probed with the oligonucleotide probe, and clones are obtained from the library that are recognized by the oligonucleotide probe so as to obtain DNA encoding another VSSC.

Specific primers derived from SEQ ID NOs 1 or 2 can be used in PCR to amplify a DNA sequence encoding a member of the VSSC family using known methods (see Innis et al. 1990). One skilled in the art will recognize that by employing such primers under high stringency conditions (for example, annealing at 50-60°C, depending on the length and specific nucleotide content of the primers employed), sequences having regions greater than 75% identical to the primers will be amplified.

More particularly, in a further embodiment the method comprises selection of a DNA molecule encoding a VSSC of an insect, or a fragment thereof, the DNA molecule having a nucleotide sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2, designing degenerate oligonucleotide primers based on regions of SEQ ID NO:1 or SEQ ID NO:2, and employing such primers in the polymerase chain reaction using as a template a DNA sample to be screened for the presence of VSSC-encoding sequences. The resulting PCR products can be isolated and sequenced to identify DNA fragments that encode polypeptide sequences corresponding to the targeted region of a VSSC.

Various modifications of the nucleic acid and amino acid sequences disclosed herein are covered by the subject invention. These varied sequences still encode a functional VSSC. The invention thus further provides an

isolated nucleic acid molecule encoding a VSSC of an insect, the nucleic acid molecule encoding a first amino acid sequence having at least 95% amino acid identity to a second amino acid sequence, the second amino acid sequence being as shown in SEQ ID NO:3. The resulting encoded VSSC is susceptible to an insecticide. The invention also provides an isolated nucleic acid molecule encoding a VSSC of an insect, the nucleic acid molecule encoding a first amino acid sequence having at least 95% amino acid identity to a second amino acid sequence, the second amino acid sequence being as shown in SEQ ID NO:4. The resulting VSSC is resistant to an insecticide.

The invention further provides isolated voltage-sensitive sodium channels of *Musca domestica*, wherein the VSSC is capable of conferring sensitivity or resistance to an insecticide in *Musca domestica*. In one embodiment, the VSSC confers susceptibility to an insecticide in *Musca domestica*, such as the VSSC encoded by the nucleotide sequence as shown in SEQ ID NO:1 (which encodes an amino acid sequence as shown in SEQ ID NO:3). In a further embodiment, the VSSC confers resistance to an insecticide in *Musca domestica*, such as the VSSC encoded by the nucleotide sequence as shown in SEQ ID NO:2 (which encodes an amino acid sequence as shown in SEQ ID NO:4). Preferably, the insecticide resistant VSSC is encoded by a nucleic acid molecule having the nucleotide sequence of a second nucleic acid molecule with one or more mutations therein, wherein the second nucleic acid molecule encodes an insecticide sensitive VSSC, and wherein the one or more mutations in the second nucleic acid molecule render the resulting voltage-sensitive sodium channel resistant to an insecticide. For example, the nucleotide sequence of the second nucleic acid molecule may encode amino acid SEQ ID NO:3, and the insecticide resistant VSSC may have that amino acid sequence with one or more differences therein

as follows: a substitution of phenylalanine for leucine at amino acid residue 1014 of SEQ ID NO:3; a substitution of isoleucine for methionine at amino acid residue 1140 of SEQ ID NO:3; a substitution of aspartic acid for glycine at amino acid residue 2023 of SEQ ID NO:3; a deletion of amino acid residues 2031-2034 of SEQ ID NO:3 (glycine-alanine-threonine-alanine); a substitution of threonine for serine at amino acid residue 2042 of SEQ ID NO:3; a substitution of alanine for valine at amino acid residue 2054 of SEQ ID NO:3; and an insertion of three amino acid residues (asparagine-glycine-glycine) after amino acid residue 2055 of SEQ ID NO:3 (between amino acid residues 2055 and 2056 of SEQ ID NO:3).

A variety of methodologies known in the art can be utilized to obtain an isolated VSSC according to the subject invention. In one method, the channel protein is purified from tissues or cells which naturally produce the channel protein. One skilled in the art can readily follow known methods for isolating proteins in order to obtain a member of the VSSC protein family, free of natural contaminants. These include, but are not limited to, immunochromatography, HPLC, size-exclusion chromatography, ion-exchange chromatography, and immunoaffinity chromatography. In another embodiment, a member of the VSSC family can be purified from cells which have been altered to express the channel protein. As used herein, a cell is said to be "altered to express the channel protein" when the cell, through genetic manipulation, is made to produce the channel protein which it normally does not produce or which the cell normally produces at low levels. One skilled in the art can readily adapt procedures for introducing and expressing either genomic, cDNA or synthetic sequences into either eukaryotic or prokaryotic cells in order to generate a

cell which produces a member of the VSSC family utilizing the sequences disclosed herein.

A VSSC as defined herein includes molecules encoding VSSCs encoded by an amino acid sequence having at least 95% amino acid identity to SEQ ID NO:3 or to SEQ ID NO:4.

Antibodies can be raised to the voltage-sensitive sodium channel. Antibodies of the subject invention include polyclonal antibodies and monoclonal antibodies capable of binding to the channel protein, as well as fragments of these antibodies, and humanized forms. Humanized forms of the antibodies of the subject invention may be generated using one of the procedures known in the art such as chimerization. Fragments of the antibodies of the present invention include, but are not limited to, the Fab, the Fab2, and the Fd fragments.

The invention also provides hybridomas which are capable of producing the above-described antibodies. A hybridoma is an immortalized cell line which is capable of secreting a specific monoclonal antibody.

In general, techniques for preparing polyclonal and monoclonal antibodies as well as hybridomas capable of producing the desired antibody are well known in the art (see Campbell 1984 and St. Groth et al. 1980). Any animal (mouse, rabbit, etc.) which is known to produce antibodies can be immunized with the antigenic channel protein (or an antigenic fragment thereof). Methods for immunization are well known in the art. Such methods include subcutaneous or intraperitoneal injection of the protein. One skilled in the art will recognize that the amount of the channel protein used for immunization will vary based on the animal which is immunized, the antigenicity of the protein, and the site of injection.

The protein which is used as an immunogen may be modified or administered in an adjuvant in order to

increase the protein's antigenicity. Methods of increasing the antigenicity of a protein are well known in the art and include, but are not limited to, coupling the antigen with a heterologous protein (such as a globulin or beta-galactosidase) or through the inclusion of an adjuvant during immunization.

For monoclonal antibodies, spleen cells from the immunized animals are removed, fused with myeloma cells, such as SP2/O-Ag 15 myeloma cells, and allowed to become monoclonal antibody producing hybridoma cells.

Any one of a number of methods well known in the art can be used to identify the hybridoma cell which produces an antibody with the desired characteristics. These include screening the hybridomas with an ELISA assay, western blot analysis, or radioimmunoassay (Lutz et al. 1988).

Hybridomas secreting the desired antibodies are cloned and the class and subclass are determined using procedures known in the art (Campbell 1984).

For polyclonal antibodies, antibody containing antisera is isolated from the immunized animal and is screened for the presence of antibodies with the desired specificity using one of the above-described procedures.

The present invention further provides the above-described antibodies in detectably labeled form. Antibodies can be detectably labeled through the use of radioisotopes, affinity labels (such as biotin, avidin, etc.), enzymatic labels (such as horseradish peroxidase, alkaline phosphatase, etc.), fluorescent labels (such as FITC or rhodamine, etc.), paramagnetic atoms, etc. Procedures for accomplishing such labeling are well known in the art, for example see Sternberger et al. 1970, Bayer et al. 1979, Engval et al. 1972, and Goding 1976.

The labeled antibodies or fragments thereof of the present invention can be used for in vitro, in vivo,

and in situ assays to identify cells or tissues which express a VSSC, to identify samples containing the VSSC proteins, or to detect the presence of a VSSC in a sample. More particularly, the antibodies or fragments thereof can thus be used to detect the presence of a VSSC in a sample, by contacting the sample with the antibody or fragment thereof. The antibody or fragment thereof binds to any VSSC present in the sample, forming a complex therewith. The complex can then be detected, thereby detecting the presence of the VSSC in the sample.

Fragments of the nucleic acid molecules encoding a VSSC are also provided, and are best defined in the context of amino acid sequence relationships among members of the VSSC sequence family and information on the function of specific VSSC domains. For example the amino acid sequence encoded by nucleotides 4648-4803 of SEQ ID NOs 1 or 2 encodes an amino acid sequence that is highly conserved among VSSC family members and is identified as the structural component forming the "inactivation gate" of sodium channels. Antibodies prepared to the polypeptide encoded by this fragment would therefore be expected to be of use as reagents capable of detecting many members of the VSSC family. Such antibodies, if introduced into cells that express VSSCs, would also be expected to modify the normal function of the VSSCs expressed in those cells. In contrast, the amino acid sequence encoded by nucleotides 3079-3852 of SEQ ID NOs 1 or 2 encodes an amino acid sequence that is less well conserved between the VSSCs of the insects *Musca domestica* and *Drosophila melanogaster*. Antibodies prepared to the polypeptide encoded by this fragment would therefore be expected to recognize selectively the VSSC from which the fragment was derived.

Also provided by the subject invention is a plasmid designated pPJI1 and deposited with the ATCC under

Accession No. 97831, as well as a KpnI/AatII restriction fragment of about 3620 bp of the plasmid designated pPJI1. Further provided is a plasmid designated pPJI2 and deposited with the ATCC under Accession No. 97832, as well as an AatII/SphII restriction fragment of about 2700 bp of the plasmid designated pPJI2. When the above two restriction fragments are ligated together at their AatII sites, the resulting nucleic acid molecule encodes a voltage-sensitive sodium channel which confers susceptibility to an insecticide in *Musca domestica*. This resulting nucleic acid molecule is also provided by the subject invention.

MATERIALS AND METHODS

Heads of newly-emerged adult house flies (NAIDM or 538ge strain) (Knipple et al. 1994) were ground to a fine powder under liquid N_2 and extracted with acid guanidinium isothiocyanate/phenol/chloroform to obtain total RNA (Chomczynski and Sacchi 1987), which was fractionated on oligo(dT)-paramagnetic beads (PolyATtract mRNA isolation system; Promega, Madison, WI) to obtain poly(A^+) RNA. Pools of first strand cDNA were synthesized using either random hexamers (Harvey and Darlison 1991) or oligo(dT) adapted for the 3'-RACE procedure (Frohman and Martin 1989). These cDNA pools were employed as templates in the polymerase chain reaction (PCR) (Saiki et al. 1988) to amplify overlapping cDNA segments spanning the entire *Vssc1* coding sequence. Mixed-sequence oligonucleotide primers employed for these amplifications comprised all possible sequence combinations encoding short (i.e., 6-8 residues) regions of amino acid conservation between the *para* gene of *D. melanogaster* and rat brain sodium channel I (Loughney et al. 1989; Knipple et al. 1991). In a few cases, mixed-sequence primers were based solely on the *D. melanogaster* sequence. Defined-sequence primers were

derived either from the previously described 309-nucleotide exon of the house fly *Vssc1* gene (Knipple et al. 1994) or from internal sequences of house fly cDNA fragments obtained by amplification with mixed-sequence primers. All primers were synthesized using an Applied Biosystems 392 instrument, deprotected using procedures provided by Applied Biosystems, desalted, and used without further purification. The sequences and designations of these primers are given in Table I. The methods and reagents employed in PCR amplifications are described elsewhere (Knipple et al. 1991; Henderson et al. 1994; Knipple et al. 1994); specific amplification conditions for each cDNA fragment were optimized by varying the annealing temperatures and extension times of the reaction. Following amplification, PCR products were separated from excess primers either by filtration of the reaction mixture through a Centricon-100 concentrator (Amicon, Beverly, MA) or by preparative electrophoresis on agarose gels, excision of the desired product, and extraction from the gel matrix (QIAquick spin column; Qiagen, Chatsworth, CA) prior to use as templates for DNA sequencing.

The DNA sequences of amplified cDNA fragments were determined by automated sequencing with an Applied Biosystems 373 instrument using fluorescently-labeled dideoxynucleotides and *Taq* DNA polymerase (PCR/Sequencing Kit; Applied Biosystems, Foster City, CA) in a modification of the dideoxynucleotide chain-termination method (Sanger et al. 1977). Sequencing of each amplification product was initiated by using the amplification primers to sequence inward from the termini, and additional primers were synthesized as needed to obtain the complete sequence of each strand. Mixed-sequence amplification primers were employed for sequencing at concentrations 10-fold higher than that used

for defined-sequence primers. All sequence ambiguities and apparent polymorphisms were resolved by performing additional multiple sequencing reactions. The full-length *Vssc1* coding sequences from the NAIDM and 538ge strains were compiled from 239 and 209 individual sequencing reactions, respectively, and were edited using the SeqEd software program (Applied Biosystems). Complete house fly *Vssc1* sequences were analyzed and compared with published sodium channel sequences using the DNASTAR software package (DNASTAR, Madison, WI).

EXAMPLE I

SEQUENCING OF THE INSECTICIDE SENSITIVE VSSC OF HOUSE FLY

As an expedient alternative to conventional iterative screenings of cDNA libraries, a sequencing strategy for the house fly *Vssc1* gene was based on the PCR amplification and direct automated sequencing of overlapping cDNA fragments (Fig. 2). The point of entry for this strategy was the 309-nucleotide exon of the house fly *Vssc1* gene identified previously from sequencing of cloned genomic DNA (Knipple et al. 1994). The use of defined-sequence primers from this region (Table I, A1 or B2) in combination with mixed-sequence primers encoding conserved amino acid sequences in either region IIS3 (A2) or the extracellular *N*-terminal domain (B1) gave cDNA fragments **A** and **B**. A second point of entry was established in homology domain IV using a pair of mixed-sequence primers (C1 and C2) to obtain fragment **C**. A primer (D2) designed from the internal sequence of fragment **C**, together with a mixed-sequence primer (D1) encoding a conserved amino acid motif in the short linker between homology domains III and IV, gave fragment **D**. A pair of

defined-sequence primers (E1, E2) based on internal sequences of fragments **A** and **D** gave the large fragment **E**, which spanned most of homology domain II and all of homology domain III. Fragment **F**, corresponding to the 5′ end of the coding sequence, was obtained using a defined-sequence primer (F2) derived from the internal sequence of fragment **B** and a mixed-sequence primer (F1) derived from a segment of the *D. melanogaster* sequence upstream from the translation start site (Loughney et al. 1989). Similarly, fragment **G**, containing the 3′ end of the coding sequence, was obtained using a defined-sequence primer (G1) derived from the internal sequence of fragment **C** and a mixed-sequence primer (G2) derived from a segment of the *D. melanogaster* sequence downstream from the stop codon (Thackeray and Ganetzky 1994).

The complete coding sequence of the *Vssc1*^{NAIDM} allele of the house fly, comprising a single open reading frame of 6318 nucleotides (SEQ ID NO:1), was determined by automated DNA sequencing using cDNA fragments **A - G** as templates (Fig. 2). This cDNA coded for a 2105-amino acid polypeptide (SEQ ID NO:3) with a predicted molecular weight of 236,671 Daltons that exhibited all of the common structural landmarks found in sodium channel α subunit genes (Catterall 1992; Kallen et al. 1993) (see Fig. 3), including four large internally homologous subdomains (I-IV), each containing six hydrophobic putative transmembrane helices (S1-S6) and a conserved sequence element between domains S5 and S6 identified as an ion pore-forming domain. The deduced *Vssc1*^{NAIDM} amino acid sequence also contained a conserved element in the S4 region of each homology domain, characterized by a repeated motif of positively-charged amino acids that are thought to form the voltage-sensing element of the channel, and a short segment of conserved sequence between homology domains III and IV that has been identified as

the channel inactivation gate (see Fig. 3). The deduced *Vssc1*^{NAIDM} protein contained 10 potential sites for *N*-linked glycosylation (Kornfeld and Kornfeld 1985), 6 of which occur in putative extracellular regions. These regions of other sodium channel α subunit sequences are also known to contain potential glycosylation sites (Catterall 1992; Kallen et al. 1993).

Vertebrate sodium channels are known to undergo functional regulation as the result of phosphorylation by cAMP-dependent protein kinases at sites in the intracellular linker between homology domains I and II and by protein kinase C at a site in the intracellular linker between homology domains III and IV (Catterall 1992; Kallen et al. 1993). The deduced *Vssc1*^{NAIDM} protein contained three potential cAMP-dependent protein kinase phosphorylation sites (Kemp and Pearson 1990) (Ser540, Ser557, and Ser628) in the cytoplasmic linker between homology domains I and II. The location of two of these (Ser540 and Ser557 of SEQ ID NO:3) corresponded to the cluster of four sites found in this region of vertebrate brain sodium channels that are implicated in sodium channel regulation (Catterall 1992). The deduced *Vssc1*^{NAIDM} protein also contained three additional potential phosphorylation sites (Ser1167, Ser1207, and Ser2097 of SEQ ID NO:3) in other putative intracellular domains. The role of these phosphorylation sites in the regulation of insect sodium channels by cAMP-dependent protein kinase is not known. The deduced house fly voltage-sensitive sodium channel protein also contained two potential sites for protein kinase C phosphorylation (Ser1191 and Ser1582 of SEQ ID NO:3) (Kemp and Pearson 1990), the latter of which is the conserved site located within the inactivation gate sequence of the cytoplasmic linker between domains III and IV. Although the conservation of this site implicates a role for protein kinase C in the regulation of insect

sodium channels, such an effect has not been demonstrated experimentally.

The deduced *Vssc1*^{NAIDM} protein was 90.0% identical to the most similar variant of the *para* gene product of *D. melanogaster* (SEQ ID NO:19) (Loughney et al. 1989; Thackeray and Ganetzky 1994) (Fig. 3). The level of sequence identity was highest (≥95%) in the *N*-terminal intracellular domain, the linker between homology domains III and IV, and homology domain IV. The level of sequence identity was lowest (73%) in the intracellular *C*-terminal domain. Alignment of the *Vssc1* sequence with 12 other sodium channel α subunit sequences found in the GenBank database showed that the *Vssc1* and *para* gene products exhibited approximately the same degree of sequence similarity as homologous sodium channel α subunit isoforms from different vertebrate species. These findings confirm and extend previous observations (Williamson et al. 1993; Knipple et al. 1994), based on fragmentary genomic DNA and cDNA sequences, of the high degree of sequence similarity between this house fly gene and the *para* gene of *D. melanogaster* and reinforce the conclusion that *Vssc1* is the homolog of *para* in the house fly.

In *D. melanogaster* (Thackeray and Ganetzky 1994; O'Dowd et al. 1995) and *Drosophila virilis* (Thackeray and Ganetzky 1995), multiple sodium channel α subunit variants, each under specific developmental regulation, are generated from the *para* gene by the alternative usage of 8 exons (designated *a-f*, *h*, and *i*) located in homology domain II and portions of the cytoplasmic linker regions on either side of this domain. Given the heterogeneity of sodium channel-encoding sequences found in these Dipteran species, it was surprising to detect only a single sequence variant among the pool of amplified house fly head cDNA fragments. The *Vssc1*^{NAIDM} sequence contained segments identical to exon *a*

and homologous (21 identical amino acids out of 24) to exon *i* of *D. melanogaster*. Recent studies suggest that both of these exons are required for the expression of high sodium current densities in embryonic *D. melanogaster* neurons (O'Dowd *et al*. 1995). In the region encoded by either exon *c* or exon *d*, the house fly sequence differs from both *D. melanogaster* sequences but is slightly more similar to exon *d* (50 identical amino acids out of 55) than to exon *c* (49 identical amino acids out of 55). The house fly sequence lacked segments homologous to *D. melanogaster* exons *b*, *e*, and *f* but contained a segment identical to exon *h*, which is a variable element found in some *D. virilis* sequences but not detected in *D. melanogaster*. The house fly $Vssc1^{NAIDM}$ sequence described is thus characterized as structurally homologous to the $a^+b^-c^-d^+e^-f^-h^+i^+$ splice variant of *D. melanogaster* and *D. virilis*. The identification of this molecular form as the predominant sodium channel sequence variant in house fly heads was unexpected because it has not been detected among the arrays of splice variants detected in whole embryos or whole adults of either *D. melanogaster* or *D. virilis*.

EXAMPLE II

SEQUENCING OF THE INSECTICIDE RESISTANT VSSC OF HOUSE FLY

The PCR amplification/ sequencing strategy summarized in Fig. 2 was also employed to determine the sequence of *Vssc1* cDNAs from heads of the 538ge house fly strain that carries the *kdr* trait. The nucleotide sequence of the VSSC of the 538ge house fly is shown in SEQ ID NO:2, and the amino acid sequence is shown in SEQ ID NO:4. The amino acid sequence of 2104 residues (SEQ ID

NO:4) encoded by the *Vssc1*^{538ge} cDNA contained 12 amino acid differences compared to that of the *Vssc1*^{NAIDM} sequence (SEQ ID NO:3) as follows: a substitution of phenylalanine for leucine at amino acid residue 1014 of SEQ ID NO:3; a substitution of isoleucine for methionine at amino acid residue 1140 of SEQ ID NO:3; a substitution of aspartic acid for glycine at amino acid residue 2023 of SEQ ID NO:3; a deletion of amino acid residues 2031-2034 of SEQ ID NO:3 (glycine-alanine-threonine-alanine); a substitution of threonine for serine at amino acid residue 2042 of SEQ ID NO:3; a substitution of alanine for valine at amino acid residue 2054 of SEQ ID NO:3; and an insertion of three amino acid residues (asparagine-glycine-glycine) after amino acid residue 2055 of SEQ ID NO:3 (between amino acid residues 2055 and 2056 of SEQ ID NO:3). A comparison of the *Vssc1*^{538ge} (SEQ ID NO:4) and *Vssc1*^{NAIDM} (SEQ ID NO:3) amino acid sequences to the *para* sequence of the Canton-S strain of *D. melanogaster* (SEQ ID NO:19) is shown in Fig. 3. The locations and amino acid sequence context of the differences are shown in Fig. 4. In Fig. 4, S refers to the NAIDM amino acid sequence (SEQ ID NO:3), and R refers to the kdr sequence (SEQ ID NO:4). Dashes indicate that the Kdr sequence has the identical residue at that position as does the NAIDM sequence. The difference labeled 1 shows amino acids 1009-1019 of SEQ ID NO:3, with the amino acid substitution at residue 1014 shown. The difference labeled 2 shows amino acids 1135-1145 of SEQ ID NO:3, with the amino acid substitution at residue 1140 shown. The difference labeled 3 shows amino acids 2018-2028 of SEQ ID NO:3, with the amino acid substitution at residue 2023 shown. The difference labeled 4 shows amino acids 2027-2038 of SEQ ID NO:3, with the deletion of residues 2031-2034 shown. The difference labeled 5 shows amino acids 2037-2047 of SEQ ID NO:3, with the amino acid substitution at residue 2042 shown. The

difference labeled 6 shows amino acids 2051-2059 of SEQ ID NO:3, with the amino acid substitution at residue 2054 shown and the insertion of three residues between 2055 and 2056 shown.

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

Table 1. Names and sequences of oligonucleotide primers used in the PCR amplification of partial *Vssc1* cDNAs.

Name	Sequence	
A1	5'-CGGTTGGGCTTTCCTGTC-3'	SEQ ID NO:5
A2	5'-GGGAATTCRAADATRTTCCANCCYTC-3'	SEQ ID NO:6
B1	5'-CCCGARGAYATHGAYCYNTAYTA-3'	SEQ ID NO:7
B2	5'-CGTATCGCCTCCTCCTCG-3'	SEQ ID NO:8
C1	5'-GGGTCTAGATHTTYGCNATHTTYGGNATG'3'	SEQ ID NO:9
C2	5'-GGGGAATTCNGGRTCRAAYTGYTGCCA-3'	SEQ ID NO:10
D1	5'-GGGTCTAGARGANCARAARAARTAYTA-3'	SEQ ID NO:11
D2	5'-TCATACTTTGGCCCAATGTC-3'	SEQ ID NO:12
E1	5'-CCCGAATTAGAGAAGGTGCTG-3'	SEQ ID NO:13
E2	5'-ACTATTGCTTGTGGTCGCCAC-3'	SEQ ID NO:14
F1	5'-CATCNTTRGCNGCNTAGACNATGAC-3'	SEQ ID NO:15
F2	5'-GATTGAATGGATCGAGCAGCC-3'	SEQ ID NO:16
G1	5'-CGTTTCTCCTTTCATATCTAG-3'	SEQ ID NO:17
G2	5'-GGAGBGGBGGNCKBGGNCKNGCTCA-3'	SEQ ID NO:18

Designation of oligonucleotide mixtures: B=G+T+C; D=G+A+T; H=A+T+C; K=G+T; N=A+C+G+T; R=A+G; Y=C+T.

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